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Intestinal Translocation Capabilities of Wheat Allergens Using the Caco-2 Cell Line

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Because intestinal absorption of food protein can trigger an allergic reaction, the effect of wheat proteins on intestinal epithelial cell permeability was evaluated and the abilities of these proteins in native or pepsin-hydrolyzed state to cross the epithelial cell monolayer were compared. Enterocytic monolayers were established by culturing Caco-2 cells, a model of enterocytes, on permeable supports that separate the apical and basal compartments. Proteins were added into the apical compartment, and the transepithelial resistance (TER) was measured; proteins that crossed the cell monolayer were detected in the basal medium by ELISA. Wheat proteins did not alter the cell monolayer. TER and Caco-2 cell viability were conserved, and the passage of dextran was prevented. Native and pepsin-hydrolyzed forms of ω5-gliadin and lipid transfer proteins were detected in the basal medium. The results suggest that these two major allergens in food allergy to wheat were able to cross the cell monolayer by the transcellular route.

KEYWORDS: **Allergy; Caco-2; crossing; intestinal barrier; LTP; ω5-gliadins; wheat proteins**

INTRODUCTION

Wheat is a highly consumed basic food, and an increase in the prevalence of food allergy to wheat (FAW) was reported during the past decade, among both children and adults (*1*). FAW induces various clinical symptoms (*2*). Whereas atopic/eczema dermatitis syndrome (AEDS), with or without asthma, occurs mainly in children, urticaria and wheat-dependent exercise-induced anaphylaxis (WDEIA) are mostly found in adults (*3*). On the basis of their solubility, wheat proteins are divided into two classes: water/salt-soluble albumins and globulins [including lipid transfer proteins (LTPs), the family of α-amylase/trypsin inhibitors, etc.] and water/salt-insoluble gliadins and glutenins, also known as prolamins, which are the major grain proteins. Gliadins are classified into α-, β-, γ-, and ω-gliadins. These ω-gliadins are composed of slow (ω1,2) and fast (ω5) ω-gliadins. Glutenins are multimeric proteins containing high molecular weight (HMW) subunits and low molecular weight (LMW) subunits. Multiple antigenic profiles in patients with FAW were found depending on age and clinical symptoms: α- and β-gliadins, LTP, and albumin/globulins appeared to be major allergens for children with AEDS with or without asthma, whereas for adults, the major allergens were ω5-gliadins in the case of anaphylaxis, WDEIA, or urticaria (*4*).

Although knowledge of the immunological basis of allergic reactions has remarkably increased over the past years, many basic issues, one of the most notable being the transport of allergens across the intestinal barrier, remain to be understood. In FAW, it has been reported that parameters such as exercise or aspirin facilitated allergen absorption from the gastrointestinal tract (*5*). Approximately 90% of nutrient absorption occurs in the small intestine. Enterocytes are specialized absorptive epithelial cells of the small intestine that have developed an apical membrane domain with a brush border structure that increases the area of absorption. The apical domain of enterocytes possesses tight junction protein complexes that seal the paracellular space between adjacent cells and thus prevent the passage of pathogens and luminal antigens. Molecules are predominantly absorbed via mechanisms such as passive diffusion (paracellular and transcellular) and carrier-mediated processes (facilitated and active) (*6*). They come in contact with the immune system associated with the gut, and they can induce allergic reactions. In several pathological situations, including food allergies, disturbance of tight junction structures resulted in increased paracellular permeability, which allows the passage of potential allergens (*7*, *8*). Recent studies also demonstrated that food allergens were able to cross the intestinal barrier by the transcellular pathway (*9*, *10*) or facilitated transport via CD23 expressed on intestinal epithelial cells (*11*).

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Varieties of cell monolayer models that mimic in vivo intestinal epithelium in humans have been developed. The Caco-2 cell line, originally derived from a human colon adenocarcinoma (*12*), was shown to undergo spontaneous in vitro enterocytic differentiation, leading in 2−3 weeks to the formation of a monolayer of highly polarized cells, with functional tight junctions, and well-developed and organized microvilli on the apical (AP) membrane. Differentiation of Caco-2 cells results in the polarized expression of brush border hydrolases (i.e., disaccharidases and peptidases) and of several transport proteins that are normally expressed in the absorptive enterocytes of the small intestine (*13*). Maintenance of Caco-2 cells on permeable filter inserts has been shown to facilitate cell differentiation and polarity, and it allows transport studies to be carried out across the cell monolayer. Over the years, Caco-2 cells have become the best-established model of the intestinal absorptive epithelium and have been extensively used to study the transport and toxicity of nutrients and xenobiotics (*14*). More recently, Caco-2 cells were used to evaluate food allergen translocation (*9*, *10*). Caco-2 cells were often used to study wheat protein effect or transport through intestinal barrier in the context of celiac disease (*15*), a severe intestinal disease resulting from intolerance to dietary wheat gluten.

The objectives of this study were to evaluate the capabilities of several wheat proteins to translocate through a Caco-2 cell monolayer and whether they could affect the paracellular permeability. As gastric digestion plays an important role in the development of food allergy (*16*) and as proteins are more or less digested in the stomach before reaching the intestinal area to be absorbed, we chose to work with native and pepsin-digested wheat proteins.

MATERIALS AND METHODS

Native and Digested Wheat Proteins. *Purification and Digest Preparation.* β-, γ-, ω1,2-, and ω5-gliadins and LMW glutenins were obtained as previously described (*17*). We used wheat LTP1 that was obtained as previously described (*18*).

The composition and purity of these fractions were controlled by electrophoresis, reverse-phase HPLC, and mass spectrometry (*17*, *18*).

Proteins (10 mg/mL in 0.1 M acetic acid, pH 2) were incubated for 2 h at 37 °C with pepsin (Sigma, P6887) at an enzyme/substrate ratio of 1:100 (w/w). The digestion was stopped by heating at 100 °C for 3 min. Digests were lyophilized and stored at room temperature. The number of free α and ϵ NH_2 functional groups in native protein (N), freeze-dried peptic digests (PD), and completely hydrolyzed proteins (T) (6 N HCl, 24 h at 105 °C) was determined as described by Frister et al. (*19*). The degree of hydrolysis (DH) was calculated according to the following equation:

$$\mathrm{DH}\ (\%) = [([\mathrm{NH_2}]_{(\mathrm{PD})} - [\mathrm{NH_2}]_{(\mathrm{N})})/([\mathrm{NH_2}]_{(\mathrm{T})} - [\mathrm{NH_2}]_{(\mathrm{N})})] \times 100$$

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Native and Digested Protein Fraction. Freeze-dried proteins, native or digested (β-, γ-, ω1,2-, ω5-gliadins, LMW glutenin, and LTP), were solubilized at 2 mg/mL in 63 mM Tris-HCl buffer, pH 6.8, containing 2% (w/v) glycerol and bromophenol blue. Samples were reduced by 5% (v/v) 2-mercaptoethanol, and 30 μg of each sample was used. Migration gel contained 15% (w/v) of polyacrylamide, and stacking gel was 6% for γ- and β-gliadins, LMW glutenins, and LTP. A gel gradient of 10−20% acrylamide was used for ω-gliadins. The migration was performed for 2 h on an SDS-PAGE gel. Gels were stained with silver nitrate.

Cell Culture. Caco-2 cells (passage 80−90) were grown in a 5% CO_2 humidified incubator at 37 °C on T25 tissue culture flasks (Sarstedt) with media containing Dulbecco's modified Eagle's medium (DMEM, Biowhittaker, Cambrex) supplemented with 10% heat-inactivated fetal calf serum (FCS), antibiotics (100 units/mL aqueous penicillin G, 100 μg/mL streptomycin (Biowhittaker, Cambrex), 2 mM L-glutamine (Biowhittaker, Cambrex), and 10 mM sodium pyruvate (Biowhittaker, Cambrex) at a pH of 7.4. Cells were passaged weekly (1:10) upon reaching confluence.

Wheat proteins were dispersed in culture medium DMEM (1 mg/mL) overnight at room temperature, filtered on 0.22 μm (PVDF, Millipore), and supplemented with 10% FCS. These filtrates were used on Caco-2 cells, and their wheat protein concentrations were determined.

Caco-2 Cell Viability. Caco-2 cells were seeded in 96-well tissue culture plates (Nunc) at a density of 1×10^4 cells/well and grown in complete culture medium for 5−7 days. Medium was replaced with 100 μL of fresh medium every 2 days. Nonproliferating, confluent cultures were treated with filtrates prepared from 1 mg/mL of native or pepsin-hydrolyzed wheat proteins or with culture medium alone (control) for 24 h in triplicate. The viability of Caco-2 cells was determined by the 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazolium bromide (MTT) method (*20*). After 24 h of incubation, the cell culture medium was replaced with 125 μL of MTT (Sigma) at 1 mg/mL in serum-free culture medium, followed by incubation for an additional 2 h at 37 °C. The formazan precipitate was dissolved by the addition of 100 μL of SDS−dimethylformamide (DMF) buffer [15% SDS (w/v) in a solution of 30% DMF, pH 4.7] and incubation overnight at room temperature. Absorbances (A) were measured at 570 nm using an automated microplate reader (ELx800UV, Bio-Tek Instruments, Winooski, VT). The percentage of viable cells was calculated using the formula

$$\%\ \text{of viable cells:}\ [(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})] \times 100$$

where A_{sample} is the absorbance of the cells treated with wheat proteins, A_{blank} is the absorbance of the solutions used, and A_{control} is the absorbance of the cells in culture medium.

Analysis of variance (ANOVA) for viability was performed to analyze the effects of proteins and their state (native or hydrolyzed) and their interactions on Caco-2 cell viability using Statgraphics Plus 3.0 software (Manugistic Inc., Rockville, MD) at a significance level of 5%.

Epithelial Barrier Function Measurements. *Transepithelial Electrical Resistance (TER) Measurements.* Caco-2 cells were plated at a density of 1×10^5 cells/well on Transwell clear polyester permeable membranes (Corning Costar), and media were changed every second day. Fully differentiated cells were obtained after 21 days of culture with the emergence of a steady-state TER. The resistance across confluent monolayers was measured using a millicell-ERS volt-ohm meter (Millipore) with "chopstick" electrodes (Millipore). Values were expressed as ohms per square centimeter ($\Omega\ cm^{-2}$), taking into account the surface area of the filter (1.13 cm^2). TER in confluent Caco-2 monolayers after 21 days exceeded 2000 $\Omega\ cm^{-2}$. The effect of wheat proteins on permeability was evaluated at day 21 by measuring TER for 8 h after the addition of filtrates prepared from 1 mg/mL native or hydrolyzed wheat proteins (β-, γ-, ω1,2-, ω5-gliadins, LMW glutenin, and LTP) to the apical side of Caco-2 cell monolayers. Each experiment was repeated two to four times according to proteins. As a positive control for tight junction opening, calcium depletion was achieved by replacing the culture medium with calcium-free phosphate-buffered saline (PBS) ($n = 5$).

Analysis of variance (ANOVA) and subsequent least significant difference (LSD) test comparison of means were performed to analyze the effects of time, proteins, and state (native or hydrolyzed) on Caco-2 cell monolayer integrity (TER).

Effect on Epithelial Permeability. The effect of wheat proteins (native or hydrolyzed) on epithelial cell permeability was evaluated by measuring the flux of 4 kDa FITC dextran (Sigma) through the Caco-2 cells monolayer. Dextran (1 mg/mL) was added to the apical compartment during the last hour of incubation of the Caco-2 cell monolayer with wheat proteins. The fluorescence of dextran in apical and basal media was measured in a microplate fluorometer (FLx800, Bio-Tek Instruments) using 485 and 516 nm filters for excitation and emission, respectively.

A standard curve was drawn to quantify 4 kDa dextran. This curve was obtained from stock solution of 4 kDa dextran dissolved at 20

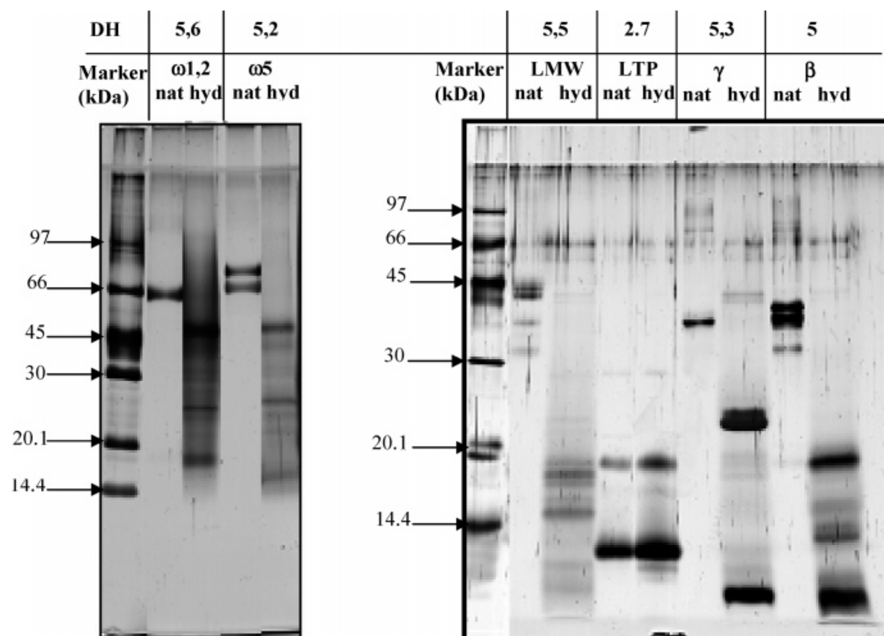

Figure 1. SDS-PAGE separation of native (nat) or pepsin-hydrolyzed (hyd) gliadin fractions (β, γ, ω1,2, ω5), glutenin fractions (LMW), and LTP1.

Table 1. Antibodies Directed against Wheat Proteins Used in ELISA Tests

name	antibody type	immunogen	peptide sequence	used to detect	ELISA type
anti-Rγ	polyclonal	repetitive peptide of γ-gliadin	QPQQPFPQ	γ-, ω-gliadins, LMW glutenins	sandwich/detection
anti-R$\alpha\beta$	polyclonal	repetitive peptide of α-, β-gliadin	PQQPYPQQPC	β-gliadin	sandwich/detection
anti-LTP	polyclonal	whole LTP		LTP	competitive/competition
anti-Rγ1	monoclonal	repetitive peptide of γ-gliadin	PQQPFPQGC	γ-, ω-, β-gliadins, LMW glutenins	sandwich/capture

mg/mL in culture medium. The quantification threshold (64.1 ng/mL) was established with the control without protein and corresponded to the mean of the control + 10 standard deviations (SD) ($n = 8$).

Transport of Wheat Proteins: Protein Quantification in Apical and Basal Media by ELISA. Wheat protein filtrates were added to the apical compartment of Caco-2 cells and incubated for 8 h. Then, apical and basal media were collected and stored at −20 °C. β-, γ-, and ω-gliadins and LMW glutenin fractions were quantified by sandwich ELISA and LTP by ELISA inhibition method using antibodies produced in our laboratory. Standard curves were obtained from stock solutions of purified gliadin and glutenin fractions dissolved at 1 mg/mL in 70% (v/v) ethanol for gliadins, in 0.05 M carbonate buffer, pH 9.6, containing 0.1% (w/v) SDS and 0.2% (v/v) 2-mercaptoethanol for glutenins, and in PBS buffer for LTP. Each determination was repeated two to five times according to the proteins. The quantification of wheat proteins was realized after solubilization and filtration to control the solubility of protein in culture medium.

Antibodies. All antibodies (polyclonal or monoclonal) directed against wheat proteins were produced in our laboratory. They are listed in **Table 1**. They were purified using protein A Sepharose (HiTap rProtein A Fast Flow, Amersham Biosciences).

Sandwich ELISA for the Quantification of β-, γ-, and ω-Gliadins and LMW Glutenin Fractions. Plates (Nunc 442404, F96 Maxisorp) were coated overnight at 4 °C, with the anti-Rγ1 (**Table 1**) monoclonal antibody (0.5 μg/well) diluted in carbonate buffer (0.05 M, pH 9.5). Remaining sites were blocked with 200 μL of 4% skim milk powder in PBS for 1 h at room temperature. Serial dilutions of wheat proteins (for standard curves) and dilutions of apical (1:1000, 1:5000) and basal media (1, 1:2, 1:5) prepared in culture medium (DMEM-10% FCS) were added (100 μL/well) for 2 h at room temperature. Polyclonal antibodies (**Table 1**; anti-Rγ for the detection of γ- and ω-gliadins and LMW glutenins or anti-R$\alpha\beta$ for β-gliadin) were added at 100 μL/well in PBS−skim milk 0.1% for 1 h at room temperature. The secondary antibody (goat anti-rabbit IgG peroxidase conjugate, Bio-Rad, Marnes la Coquette, France; 100 μL/well) was incubated for 1 h at room temperature. After each step, wells were washed three times with 200 μL of PBS containing 0.05% (v/v) Tween-20. Detection was performed with 100 μL of *O*-phenylenediamine (Sigma, St. Louis, MO) at 0.4 mg/mL and H_2O_2 0.03% in 0.05 M sodium citrate, pH 5.5, for 30 min at room temperature and stopped with 25 μL of 4 N H_2SO_4. The absorbance was read at 490 nm using the automated ELISA reader ELx800UV with KC4 software package (Bio-Tek Instruments). The quantification threshold was established with the control without protein and corresponded to the mean of the control + 10 SD ($n = 4$).

Inhibition ELISA Assay for LTP Quantification. For inhibition ELISA tests, plates were coated overnight at 4 °C, with the LTP protein (0.1 μg/well) diluted in carbonate buffer (0.05 M, pH 9.5). Polyclonal antibody anti-LTP (**Table 1**) was preincubated for 2 h at 37 °C with serial dilutions of LTP for reference curve or with serial dilution of apical and basal media samples in culture medium. The antibody−antigen mixtures (100 μL) were added to plates coated with LTP. We quantified proteins only in the linear zone of the curve.

RESULTS

In Vitro Pepsin Digestion of Purified Wheat Proteins. The purity of native wheat protein fractions (β-, γ-, ω1,2-, ω5-gliadins, LMW glutenins, and LTP) and their pepsin digests was analyzed by SDS-PAGE (**Figure 1**). For all purified wheat proteins, no cross-contamination was observed. The degrees of hydrolysis were low, around 5−6% for gliadins and 3% for LTP.

For all prolamin fractions, native proteins were no longer observed after pepsin digestion.

In SDS-PAGE, native ω-gliadins migrated at a molecular mass (MM) around 66 kDa: two bands for ω5-gliadins (67−70 kDa) and one for ω1,2-gliadins (66 kDa). Pepsin digests of the two ω-gliadin fractions presented similar electrophoretic profiles, with polypetides migrating between 45 and 14 kDa, with three major bands occurring at 45, 25, and 15−17 kDa.

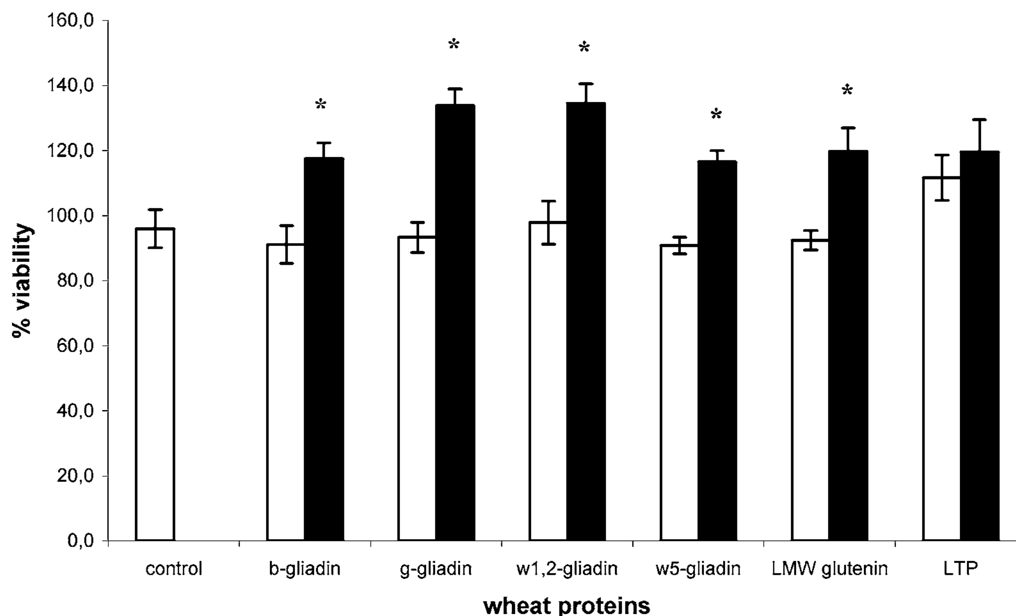

Figure 2. Effect of wheat proteins on Caco-2 cells viability after 24 h of incubation. Influence of protein state (native, □; hydrolyzed, ■; $n = 3$) was analyzed by ANOVA and Student's *t* test. $*$, $p < 0.05$.

Native γ-gliadins migrated at MM 40 kDa and were hydrolyzed by pepsin into peptides with two major bands at 25 and 5 kDa. Native β-gliadin fraction comprised three bands detected around 40 kDa, and pepsin digestion produced polypetides migrating between 20 and 5 kDa with three major bands at 20, 15, and 5 kDa.

Native LMW glutenins comprised several subunits with MM ranging from 45 to 30 kDa. All subunits were hydrolyzed by pepsin into polypetides migrating between 25 and 5 kDa, with two main bands (18 and 15 kDa).

Native LTP1 migrated around 10 kDa; however, another band was visible at 20 kDa, corresponding to a dimer of LTP1 (*21*). This dimer seemed to persist despite reducing conditions. After pepsin treatment, the two bands of native LTP1 persisted, and we did not observe any peptides on the gel despite a DH of 3%. Maybe this hydrolysis induced some very small peptides that could not be observed on the gel.

To summarize, we observed a low DH for all wheat proteins. The digestion was sufficient to degrade all native prolamins; however, polypeptides of relatively high MM were conserved (45, 25, 20 and 15 kDa). On the contrary, native LTP1 was able to resist pepsin hydrolysis, and no peptides could be observed.

Effect of Purified Wheat Proteins on Caco-2 Cell Viability. Results from the MTT assay (**Figure 2**) revealed that native wheat proteins did not alter the viability of Caco-2 cells after 24 h of incubation. An increase in the percentage of viability with all hydrolyzed proteins, especially with γ-gliadins ($p = 0.0005$) and ω5-gliadins ($p = 0.0005$), was observed except for LTP ($p = 0.32$). Hydrolyzed wheat proteins seemed to induce proliferation of Caco-2 cells, being beneficial for cell survival, and were potentially metabolized by cells.

Wheat proteins in native or hydrolyzed form did not reduce the viability of confluent Caco-2 cells.

Effect of Purified Wheat Proteins on Caco-2 Cell Monolayer Permeability. During monolayer establishment (21 days), TER increased and became stable at the day 16 at approximately 2000 Ω cm^{-2}.

The influence of incubation of wheat proteins on Caco-2 cells permeability is shown in **Figure 3**. TER slightly increased during the 8 h of incubation with native or hydrolyzed wheat proteins ($p < 0.0001$). As a positive control for tight junction opening, calcium depletion by replacing the culture medium with PBS effectively induced a 20% reduction of the TER in the first 3 h. The absence of a TER decrease with wheat proteins confirmed the data previously obtained with the MTT test, showing that wheat proteins did not alter the monolayer of Caco-2 cells.

The influence of the type and state of wheat proteins on Caco-2 cells permeability is presented in **Figure 4**. The mean TER measured during 8 h of incubation more or less increased depending on wheat proteins ($p < 0.0001$): β-, γ-, and ω1,2-gliadins and LMW glutenin showed slightly increased TER (about 2%), whereas the increase was higher with LTP and ω5-gliadins (about 7−8%). We noticed a higher increase of TER with pepsin digest ($p = 0.0148$), but this difference was significant only for LMW glutenin, ω5-gliadin, and LTP (LSD test).

To confirm the absence of a wheat protein effect on epithelial cell permeability, we measured the flux of 4 kDa FITC dextran through the Caco-2 cell monolayer. No dextran was found in the basal compartment with a limit of quantification of 64.1 ng/mL established according to standard curve. Wheat proteins in native or hydrolyzed state had no effect on epithelial cell permeability. They provoked no paracellular transport and conserved the integrity of cell monolayer.

Quantification of Wheat Proteins in Apical and Basal Media of Inserts. The ability of wheat proteins to translocate across the Caco-2 cell monolayer was studied by quantifying the presence of wheat proteins in the basal compartment. For the detection and quantification of proteins, we performed sandwich ELISA for β-, γ-, and ω-gliadins and LMW fractions and inhibition ELISA assay for LTP. Standard curves were different between the native and hydrolyzed states of wheat proteins except for LTP (**Figure 5**). Indeed, the thresholds of quantification were lower for native than for hydrolyzed proteins except for LTP and ω5-gliadins. These observations demonstrated that pepsin digestion affected the recognition of prolamins by antibodies but not that of LTP. This related to the observation by SDS-PAGE (**Figure 1**) that all proteins except

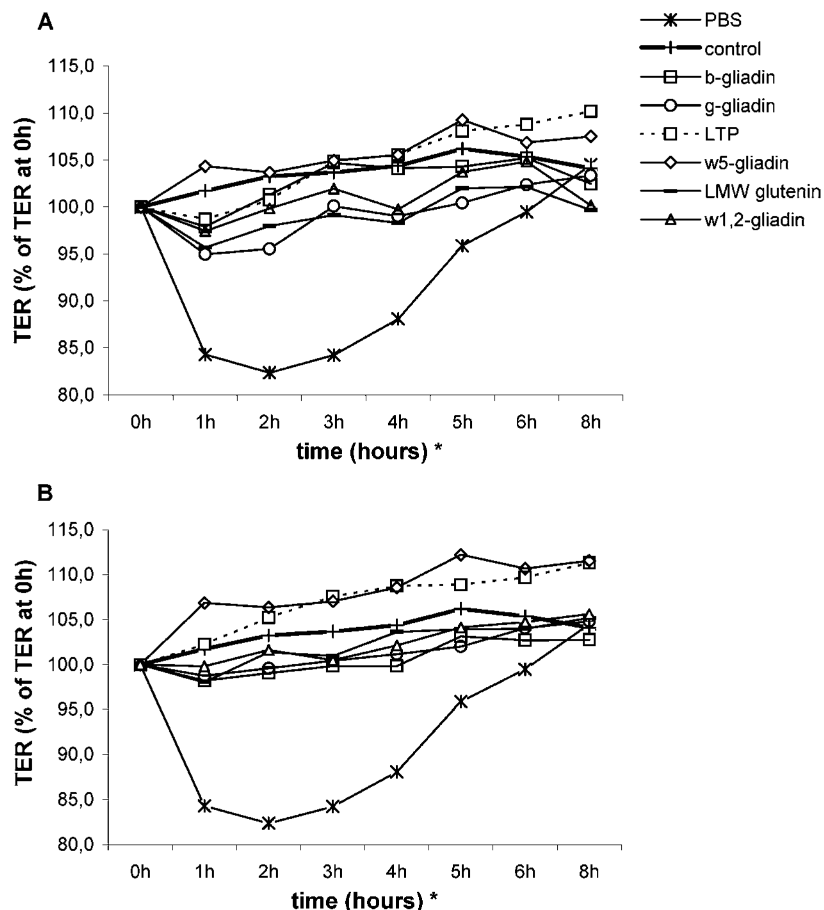

Figure 3. TER evolution during the 8 h of incubation with native (**A**) or hydrolyzed (**B**) LMW glutenin fraction, β-, γ-, ω1,2-, and ω5-gliadins, and LTP. Influence of time was analyzed by ANOVA and subsequent comparison test (LSD test). *, $p < 0.05$.

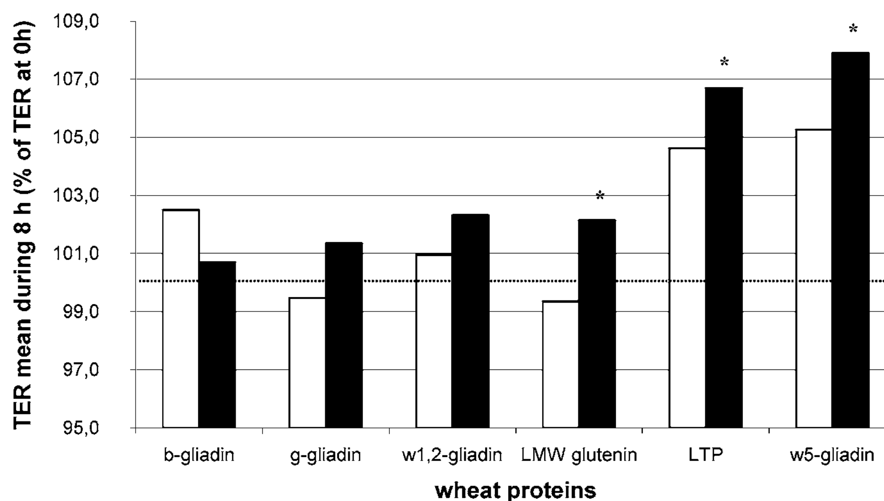

Figure 4. Effect of native (□) and hydrolyzed (■) wheat proteins on the TER of a Caco-2 cell monolayer. TER values corresponded to the mean of all TER (percent of TER at 0 h) during 8 h of incubation ($n = 8$). Influence of wheat proteins and their state was analyzed by ANOVA analysis. $*$, $p < 0.05$.

LTP were digested by pepsin into polypeptides. The quantification threshold was between 0.003 and 0.06 μg/mL for all wheat proteins except for hydrolyzed (about 1 μg/mL) and native (about 0.15 μg/mL) β-gliadins and hydrolyzed LMW glutenins (about 0.3 μg/mL). The better recognition of γ- and ω1,2-gliadins was due to the capture antibody anti-Rγ1, which corresponded exactly to the repetitive motif present in these gliadins, whereas the repetitive motifs of ω5- and β-gliadins and LMW glutenins are slightly different.

Wheat proteins were quantified after solubilization in DMEM at 1 mg/mL and filtration; they were also quantified in apical and basal media after 8 h of incubation on Caco-2 cells (**Table 2**). We observed a low protein concentration in filtrates of native β-, γ-, ω1,2-gliadins and LMW glutenin (from 50 to 200 μg/mL). Such a low solubility of native proteins in the culture medium may have partially affected the study of their transport. For the other proteins and all hydrolyzed fractions, except ω1,2-gliadin, protein concentration in filtrates were between 700 and 1000 μg/mL. Similar concentrations were found in apical media and filtrates for all fractions.

In basal media, only native forms of ω5-gliadin and LTP were detected at 0.2 and 0.3 μg/mL, respectively. Higher

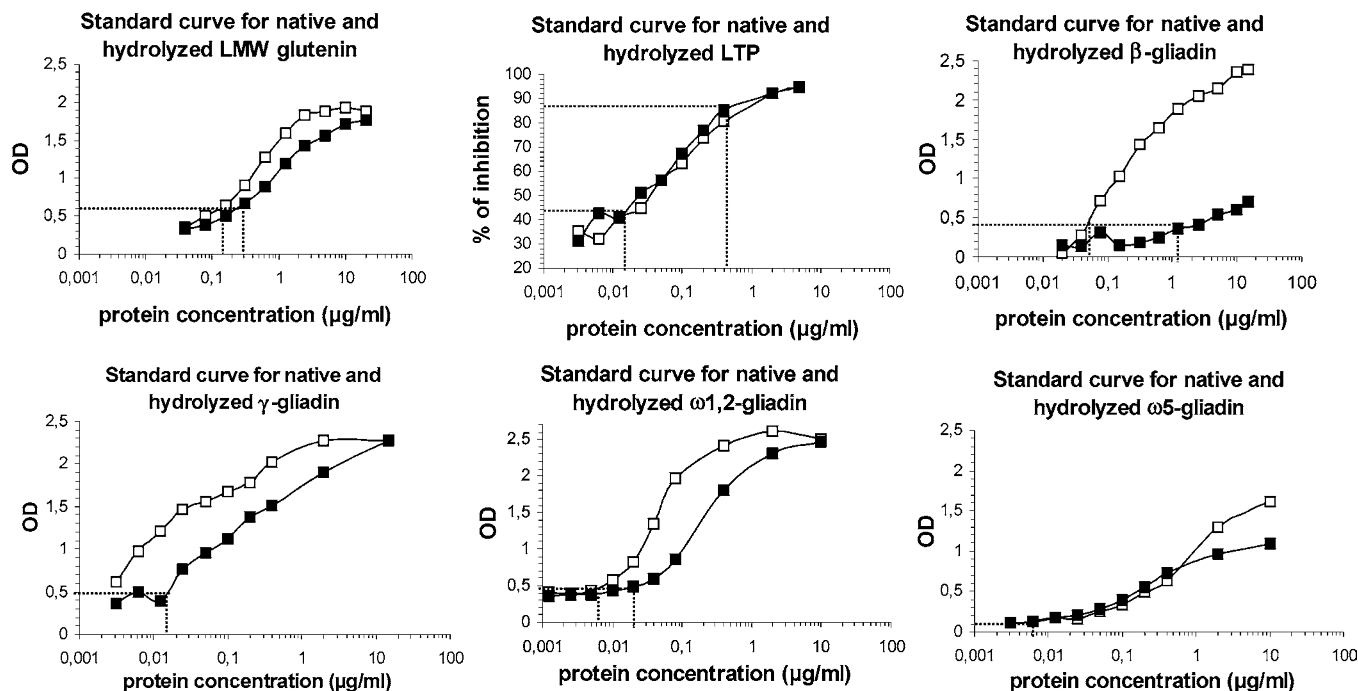

Figure 5. Analysis of wheat protein translocation across Caco-2 cells monolayers: standard curves were obtained by sandwich (β-, γ-, ω1,2-, and ω5-gliadins, LMW glutenin) or inhibition (LTP) ELISA for wheat protein quantification in native (□) or hydrolyzed (■) form.

Table 2. Analysis by ELISA of the Concentration of Wheat Protein in Native (nat) or Hydrolyzed (hyd) Form in the Culture Media of Caco-2 Cells

wheat protein (nat or hyd)	filtrate (μg/mL)	apical medium (μg/mL)	basal medium (μg/mL)
ω5-gliadin nat	755	673	0.2
ω5-gliadin hyd	882	712	3.4
β-gliadin nat	201	101	nd[a]
β-gliadin hyd	944	876	nd
γ-gliadin nat	32	32	nd
γ-gliadin hyd	700	921	nd
ω-gliadin1,2 nat	274	308	nd
ω-gliadin1,2 hyd	354	415	nd
LMW glutenin nat	153	127	nd
LMW glutenin hyd	1242	1086	nd
LTP nat	1423	1197	0.3
LTP hyd	1016	708	0.2

[a] Not detected.

amounts of hydrolyzed ω5-gliadin were measured (3.4 μg/mL) in this compartment. Hydrolyzed LTP was detected at a similar amount as the native form (0.2 μg/mL), probably linked to the absence of digestion by pepsin. The hydrolyzed fractions of β-, γ-, and ω1,2-gliadins and LMW glutenins were not detected.

DISCUSSION

It is generally thought that food allergens are rather resistant to proteolysis, thus reaching the intestine more or less unaltered, where they elicit the immune response (*22*). Wheat proteins were relatively resistant to pepsin with low DH (3−6%) after 2 h of digestion by pepsin but with no persistence of native form of proteins except for LTP. The resistance of LTP to pepsin digestion, in agreement with Asero et al. (*23*), contributes to the explanation of why it is a potentially severe food allergen. Our results suggest that pepsin digestion of gliadins induces the production of large peptides (between 45 and 20 kDa) containing several repetitive motives, allowing the simultaneous binding of two antibodies specific for the repetitive domain of γ- and ω1,2-gliadins. This suggestion agrees with Masson et al. (*24*), who demonstrated that pepsin cleavage of γ-gliadins induced large polypeptides of 28 kDa, corresponding to the repetitive domain. For the first time, similar results were obtained here for ω-gliadins, where fragments obtained by pepsin digestion were large. These fragments were even larger than those from the other prolamins. Indeed, these proteins possess a longer sequence that is entirely composed of a repetitive domain. We can conclude that all of the tested wheat protein fractions were rather resistant to gastric digestion and can potentially act on intestinal epithelium to activate the immune system.

Several studies were carried out on the effect of gliadins on intestinal epithelium, as these proteins are involved in celiac disease. In this pathology, ingestion of gluten leads to damage of the small intestine, with avillous flattening and crypt hyperplasia, and to a slight increase of inflammatory infiltrate in both the epithelium and lamina propia (*25*). In our study, contrary to some publications (*15*, *26*, *27*), no toxicity of wheat proteins (native or hydrolyzed) was observed. In the case of Brazil nut allergy, Moreno et al. also reported no effect of native or digested 2S albumin allergen on Caco-2 cell viability (*9*). Purified wheat proteins had no effect on Caco-2 cell viability, whereas Giovannini et al. (*27*) showed that digestion of gliadins induced apoptosis through the CD95/Fas apoptotic pathway. However, culture conditions of Caco-2 cells and gliadin digests were different, as Gioviannini et al. worked with nonconfluent Caco-2 cells and one peculiar fraction of peptic-tryptic digests. We found a slight increase of TER during the 8 h of incubation, and no dextran flux was observed with native or digested wheat proteins. This disagrees with Sander et al. (*15*), who found that gliadins altered barrier function almost immediately, with a 40% decrease of TER and an increase of permeability to 4 kDa dextran. The major difference between experiments is the digestion type: we used peptic digests instead of peptic-tryptic digests. We can hypothesize that trypsin digestion may allow the production of toxic gliadin peptides for intestinal epithelium.

To test this hypothesis, the effects of peptic-tryptic digests of gliadin extract or β- and γ-gliadins on Caco-2 cell viability and TER were studied. However, in our conditions, peptic-tryptic digests did not decrease the viability and did not increase the permeability of Caco-2 cells monolayer (results not shown).

In the case of food antigens, two different mechanisms (paracellular and transcellular) of transport across the intestinal epithelium enterocytes were described. The paracellular way, the diffusion of food antigens through the tight junctions, is negligible under physiological conditions (*7*). The process of food antigens by the transcellular transport involves two functional pathways, one minor direct pathway without degradation and another major lysosomal degradative pathway (*7*).

To screen for the ability of wheat proteins to cross the monolayer, quantitative ELISA tests were elaborated to detect native or hydrolyzed proteins in the basal compartment of Caco-2 monolayers using specific antibodies. These tests may have some limitations related to the reactivity of antibodies toward proteins hydolyzed by pepsin and/or cellular processing. These antibodies are peptide-specific, which restricted their reactivity but may allow for the detection of proteins in the denatured state. The native forms of ω5-gliadin and LTP were able to cross the monolayer of Caco-2 cells and be detected in the basal compartment by ELISA. Pepsin digestion of ω5-gliadin enhanced its ability to pass through the Caco-2 cell monolayer. Approximately the same percentage of protein transport across Caco-2 cells was observed for digested ω5-gliadin (0.48%) as for native β-lactoglobulin (0.33%) (*10*). As the TER was not disturbed and no dextran flux was detected, it is likely that the passage of proteins from apical to basal compartment occurs through a transcellular route rather than through paracellular diffusion. This is also attested by the time period (8 h) necessary to detect the proteins in the basal medium. The capacity of enterocytes to internalize and transport small amounts of intact proteins or large fragments with antibody binding capacity, which could be involved in the immunological sensitization to food allergens, has been previously reported (*9*, *28*). The transcellular pathway has been previously reported in the uptake of bovine milk allergens, β-lactoglobulin and α-lactalbumin, through enterocytes (*10*, *29*). It is likely that ω5-gliadins and LTP were transported by the direct pathway with an incomplete degradation. It is also noteworthy that these two proteins are known as important allergens. ω5-Gliadin is described as a major allergen for patients suffering from WDEIA (*4*, *30*). Several immunodominant epitopes of ω5-gliadin and a consensus motif of the type QQX1PX2QQ (X1 being L, F, S, or I and X2 being Q, E, or G) were identified for these patients (*30*, *31*). LTP is an important allergen for children with AEDS (*4*). LTP1 is a small protein of about 9 kDa. This could explain its ability to cross epithelial barrier in a native form and be allergenic. We project to identify polypeptides from ω5-gliadin and LTP1 that were able to cross the barrier using proteomic tools and, in the case of ω5-gliadins, to compare them with those identified as allergenic epitopes.

More physiological systems such as M cell (*10*) and intestine from sensitized rat (*32*) had also demonstrated the transcellular transport of allergens. Both cell types (Caco-2 cell and M cell) were able to transport β-lactoglobulin (*10*), which confirmed the Caco-2 cell model relevance. Specific sensitization was shown to enhance the initial uptake and transcytosis of antigen across intestinal epithelium, and following the activation of immune system (mast cell), antigen transport was further enhanced by penetration through the paracellular pathway (*32*). This observation demonstrated the importance of immune system activation to produce molecules such as inflammatory cytokines to open tight junctions and to allow allergen transport amplification by the paracellular pathway.

The other wheat proteins were not detected in the basal media by ELISA, maybe because they were degraded by brush border enzymes or they were transported in cells by the major lysosomal degradative pathway. In this study, we tested only the free transport of wheat proteins across the intestinal barrier, but some studies show that a facilitated transport of IgE–allergen complexes via CD23-bearing intestinal epithelial cells exists (*11*). IL-4 up-regulates CD23 expression in intestinal cells and enhances transepithelial antigen transport (*11*). This pathway even seems to protect allergens from degradation during transcytosis (*33*). It would be interesting to test this translocation pathway for wheat proteins that were not detected in basal media (β-, γ-, and ω1,2-gliadins and LMW glutenins).

To conclude, Caco-2 cell is a good model to study allergen effects and their ability to translocate through intestinal cell monolayer. This model allows us to demonstrate that two important allergens, ω5-gliadin and LTP, were able to cross this barrier, probably by the transcellular route, and kept their immunologic recognition by antibodies. Pepsin digestion facilitated this transcellular transport of ω5-gliadins.

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